Biologic Activity of Purified Cotton Bract Extracts in Man and Guinea Pig

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Purified aqueous extracts of cotton bract induce acute airway constriction in healthy volunteers never before exposed to cotton bract. The response is similar to that of textile workers who inhale cotton dust. Approximately 60% of volunteers respond to bract extract with significant decreases in lung function, and these volunteers show an increased number of lymphocytes present in their lungs. Following inhalation of bract, the percent of polymorphonuclear leukocytes increases. Macrophages obtained by bronchoalveolar lavage from volunteers pre-challenged with bract extract release increased amounts of chemotactic factor and superoxide anion. Efforts to detect release of histamine and leukotrienes in volunteers following challenge with bract show no increase in urinary histamine and no significant release of leukotrienes in lung lavage fluid. Purified extracts exhibit chemotactic activity in vitro. They also contract guinea pig ileal longitudinal muscle in vitro. This preparation contains mast cells but no basophils, and the H-1 blocker, mepyramine blocks the contraction. Purified bract extracts contain no histamine or endotoxin but other contractors of smooth muscle may be present. The purified extract exhibits spectral, fluorescent, and radioimmune assay properties similar to a leukotriene B-like component. Cotton bract appears to have direct as well as cell-mediated activities.

Introduction

The processing of cotton into yarn creates airborne dust of inhalable size which causes byssinosis, the occupational lung disease of the textile worker. The response to cotton dust is characterized by acute symptoms of chest tightness, shortness of breath, and cough accompanied by a decrease in lung function (1). This response is a "Monday" response occurring, at least initially, only on the first day back to work after a weekend or other absence. The effect appears to be more than a mechanical irritation effect and it does not appear to involve an antigen–antibody immunological response (2). Rather, dust contains a potent nonantigenic airway constrictor. The chemical identity of this constrictor is unknown, as is its mechanism of action. Dust may contain a constrictor which reacts directly with airway smooth muscle, the constrictor's action may be indirect via stimulation of lung cells to release mediators of smooth muscle contraction, or both actions may be involved.

The acute airway constrictor response can be reproduced in the laboratory in human volunteers who inhale aqueous extracts of cotton bracts (3). Bract (modified leaf structure surrounding the stem at the base of the cotton boll) is a principal plant trash component in cotton mill dust (4). The response is seen in healthy persons never before exposed to cotton dust. In previously reported studies, we have used this inhalation challenge testing of volunteers as a bioassay procedure to determine airway constrictor activity of purified bract extracts in attempts to purify and identify this bract constrictor agent (5). Although the identity is not yet known, it was shown that neither the histamine content nor the endotoxin content in bract extracts could account for the airway constriction observed (6). However, other possibilities exist and these are discussed here. The in vitro contraction of guinea pig smooth muscle by bract extracts is also discussed.

Challenge studies with volunteers inhaling bract extracts have now been used in combination with bronchoalveolar lavage in attempts to learn more about the lung's response to cotton bract. Results of lung cell population, chemotaxis, and release of mediators and other factors in vivo are presented. Our findings are discussed

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in terms of a direct versus an indirect action of airway constriction by cotton bract extracts.

Materials and Methods

Preparation of Cotton Bract Extracts

Extracts were prepared as described previously (7). Dried bracts (frost killed) were hand picked just prior to harvest from cotton fields in the Lubbock, Texas area, and stored at room temperature. Crude extracts were prepared by a simple aqueous extraction using 1 g bracts/ 6 mL water. Purified extracts were prepared by methanol precipitation of contaminants followed by anion-exchange chromatography on DEAE-Sephacel. Extracts were freeze-dried and stored at –4°C. For inhalation challenge by our volunteers, each extract was reconstituted with water at a concentration equivalent to the standard crude extract. This insured that for challenge purposes components were not concentrated as purification progressed.

Assay for Acute Airway Constriction in Humans

From area universities, we recruited healthy volunteer subjects (no respiratory symptoms, no history of asthma), ages 18 to 39 years, males, females, smokers and nonsmokers. Airway constrictor effects of the bract extracts were assayed by comparing lung function values obtained from recordings of partial and maximum expiratory flow-volume (PEFV, MEFV) curves before and at 30-min intervals for a 2.5–3 hr period following a 10 min inhalation of the aerosolized extract (3). Forced expiratory volume in 1 sec ($FEV_{1.0}$) was measured from the MEFV curve and instantaneous flow rate at 40% of remaining vital capacity [MEF40%(P)] was measured from the PEFV curve. MEF40%(P) is the more sensitive indicator of airway constriction and allows us to detect a measurable response without the need to induce severe constriction and corresponding discomfort for our volunteers. All volunteers were screened with our standard crude bract extract to determine their reactivity to cotton extracts. Those who responded with a 20% or greater decrease in MEF40%(P) (approximately 60% of the volunteers) were classified as responders. Nonresponders were those whose MEF40%(P) decreased less than 20%. Extracts were administered as an aerosol of less than 1 μm diameter generated by use of a Dautrebande D–30 nebulizer.

Bronchoalveolar Lavage Procedure

Lavage was performed as previously described (8). Briefly, the nose and upper airway were anesthetized with topical 4% lidocaine and the bronchofiberscope was passed transnasally or orally. Minimal amounts of lidocaine (1%) were used endobronchially to suppress cough. The bronchoscope was advanced into a subsegment of the lingula until it reached maximum penetration. Aliquots of sterile 0.9% saline (50 mL each) were alternately instilled and aspirated until 300 mL has been instilled. Recovered fluid was filtered through two layers of gauze mesh to remove gross mucus particles and centrifuged (4°C, 500g, 10 min) to pellet cellular elements. The cellular elements were washed once in modified Hanks solution (Ca^{2+}- and Mg^{2+}-free, Grand Island Biologicals, Grand Island, NY) and resuspended in modified Hanks and put to culture (9).

Chemotactic Assay

Human blood polymorphonuclear leukocytes were used for measurement of chemotactic activity. A leading front method was adapted from Zigmond and Hirsch (10). Blind well chemotactic chambers were used with a double membrane of 5 μm polycarbonate over 3 μm nitrocellulose (11).

Superoxide Anion Assay

Lung macrophage release of superoxide anion (O_2^-) into the culture medium was measured by superoxide dismutase-inhibitable cytochrome c reduction (12,13).

Leukotriene Assay

Bronchoalveolar lavage fluid was assayed for presence of leukotrienes C and B by the radioimmune assay performed by Dr. A. W. Ford-Hutchinson, Merck Frosst Laboratories, Canada. The lavage fluid was stored at – 79°C and packed in dry ice during transit.

Histamine Assay

Histamine content of bract extracts and urine was determined by the HPLC method of Wall et al., which utilizes post-column derivatization with o-phthaldehyde for detection (14). A CN (10 μm) column was used.

Guinea Pig Smooth Muscle Strips

Lung parenchymal and ileal longitudinal muscle strips were obtained from male Hartley guinea pigs (300–500 g). The parenchymal strips were prepared by cutting a strip from the outer edge of a lung lobe which measured about 3 × 3 mm sq and 3 to 4 cm in length (15). Longitudinal muscle was dissected from guinea pig ileum by the procedure of Rang (16). Both lung strips and ileal strips were suspended in a 5 mL tissue bath containing oxygenated Krebs bicarbonate solution at 37°C. The tissues were attached to force displacement transducers (FT03C) and responses recorded on a Grass polygraph. A passive force of 0.5 g was applied to the lung strips and 2 g to the ileal longitudinal strips for 20 min prior to addition of histamine (0.5 μg/mL) as the positive control. After wash-out of the histamine, tissues were challenged with bract extracts.

Results and Discussion

Human Response to Cotton Bract Extract Challenge

To a challenge with a standard extract of cotton bract, approximately 60% of healthy naive volunteers responded with decrements in lung function showing the wide spread nature of this effect. These responders showed a wide response range for each individual with a mean decrease in the flow value, the MEF40%(P), of 31% and in $FEV_{1.0}$ of 8%. Standard extracts of cotton dust rather than bract caused similar decrements. The decrements in $FEV_{1.0}$ reported for mill workers over a work shift are also similar, despite the differences in challenge procedures: workers receive a continual exposure of dust over several hours, whereas our volunteers receive an aerosol exposure for only 10 min. Naive volunteers who respond appear not to be subclinical asthmatics with hyperreactive airways as we found no correlation between an individual's response to histamine and his or her response to bract extract (17). However, a positive correlation was found between responsiveness to methacholine and to cotton bract (18).
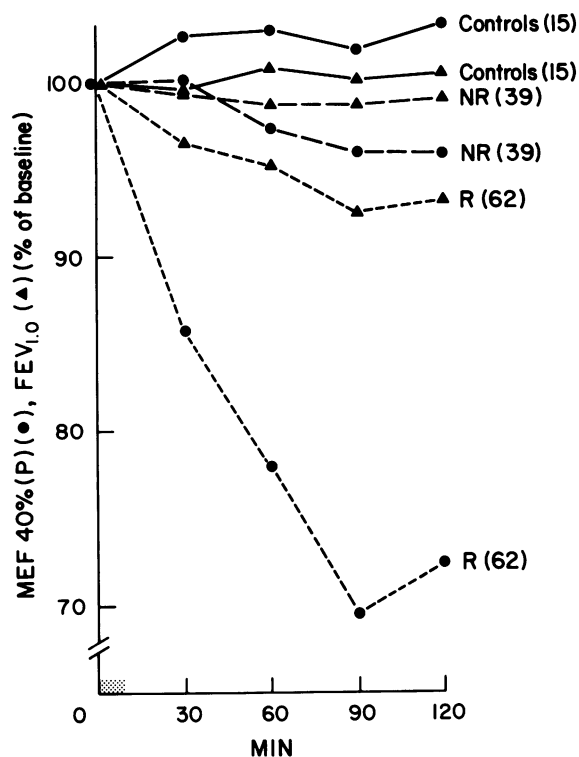

FIGURE 1. Time-course of response to standard crude bract extract. Plots of (○) mean MEF40%(P) and (▲) $FEV_{1.0}$ responses to the crude extract aerosol as a function of time after the start of aerosol inhalation (0–10 min; shaded bar). The subjects were arbitrarily divided into two groups; the nonresponders (—) those who had a MEF40%(P) decrease of less than 20%; and the responders (- -), those who had a MEF40%(P) decrease of more than 20%. Number of subjects in each group in parentheses. From Buck (7).

An examination of the numbers and kinds of cells obtainable by bronchoalveolar lavage which are present in the lungs of responders and nonresponders before they have had any exposure to cotton dusts or cotton extracts showed that responder's lungs had significantly more lymphocytes present (9.7%) than did lungs of nonresponders (2.8%) (19). The relative percent of other cells, alveolar macrophages, polymorphonuclear leukocytes, esinophils was not different. Lymphocytes are not known to be directly involved with contraction of smooth muscle, but they are known to release substances which in turn can stimulate other luminal cells.

The response to cotton bract is unlike the rapid response caused by histamine or methacholine. The bract effect is characterized by a delay of 90 to 120 min before reaching maximum (see Fig. 1), and then slowly diminishes over the next several hours (20). It is not known if this delay results from time needed for the active component(s) in the bract extract to penetrate into the area of the smooth muscle or if the mechanism of airway constriction is via the stimulation of certain lung cells and their subsequent release of mediators which then act on airway smooth muscle.

Under appropriate stimuli, polymorphonuclear leukocytes are known to migrate into the alveolar spaces, and these cells are known to release mediators of smooth muscle contraction. Bouhuys et al. reported some years ago an increase in the numbers of circulating leukocytes in blood of workers over the work shift (21). In this instance he was studying Spanish hemp workers. Others have reported observing migration of leukocytes into the nasal passages of workers exposed to cotton dust. We looked for an increase in the numbers of circulating leukocytes in volunteers following inhalation challenge with cotton bract extracts. Blood was drawn before and again 60 to 90 min following the inhalation. The data for blood leukocyte count versus decrease in lung function is shown in Figure 2. There is little increase in circulating leukocytes seen. Either this phenomenon is not associated with the acute airway constriction or perhaps a longer time (beyond where maximum constriction occurs) is needed for full manifestation of the effect.

Bronchoalveolar lavage offers a direct look at the possible changes in cell population within the lung itself following exposure to cotton bract extracts. Accordingly, volunteers were challenged by inhalation of bract extract; 60 min following this challenge, bronchoalveolar lavage was performed and cell counts made on the lavage fluid (22). Comparisons were made between responders and nonresponders prechallenged with bracts, and between these and saline-challenged, or unchallenged controls. The differential cell counts of the lavage fluid continued to show that responders had a higher percent content of lymphocytes than did nonresponders, as was shown previously with unchallenged responders and nonresponders.

The percent of polymorphonuclear leukocytes was found to be increased following bract challenge. Responders increased compared with nonresponders, and
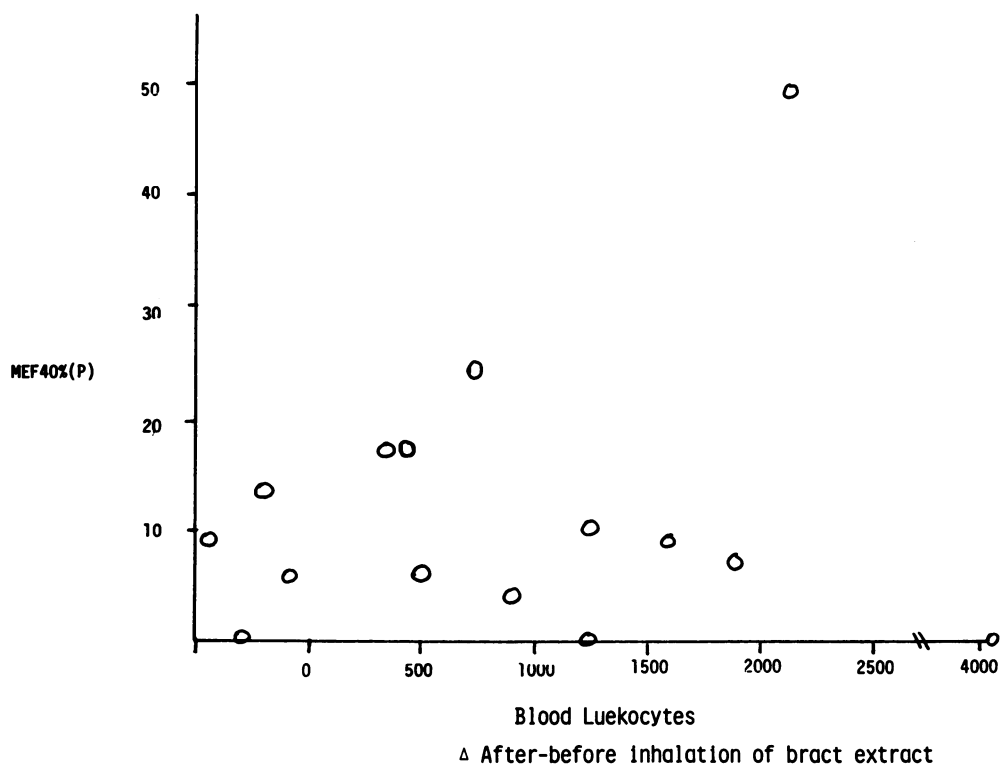
FIGURE 2. MEF40%(P) vs. blood leukocyte count before and after bract challenge. Plot of % decrease in MEF 40%(P) 90 min following inhalation of the bract extract vs. leukocyte count, 60–90 min after inhalation minus before inhalation. Each open circle is the result from one individual.

moreover, this increase in PMN correlated ($p<0.02$) with extent of the individual's airway constrictor response. At the time of lavage, about 90 min after inhalation of the extract, the total cell count in the lavage fluid was not increased.

Migration of PMNs into the alveolar spaces could result either from a direct chemoattracting activity of the bract extract itself or through bract stimulation of lung cells to release chemoattractants, or both. To test for direct chemotactic activity of bract extracts, we used leading front experiments where chemotactic activity was estimated by counting migration of human blood PMN through a membrane towards the bract extract. Table 1 shows the results of a typical experiment using our standard crude extract of bracts. This extract is a PMN chemoattractant. Maximum migration was observed at a 1:100 dilution of the extract. Medium without bract extract was used as a negative control; zymosan-activated serum, a potent chemoattractant, was used

as the positive control. The bract extract exhibited 42% of the activity of the zymosan-activated serum. Purified bract extracts, including those which contained no endotoxin, gave similar results. Additional controls were also run to show that the PMN migration observed was not due to random movement. In the checkerboard set-up with bract extract placed both above and below the membrane, no migration was observed.

Bract extracts were shown also to cause release of chemotactic factor from alveolar macrophages (22). Volunteers who had been prechallenged with bract extract underwent bronchoalveolar lavage approximately 60 min following the bract challenge. The lung cells recovered in the lavage were separated from the fluid portion and subsequently washed several times to insure that all traces of the bract extract which had been inhaled was removed before the cells were put to cell culture. These cells were shown to release chemotactic activity into the culture medium (see Table 2). The increased release of

Table 1. Bract-derived PMN chemoattractant.

Bract, dilution of SCE[a]	n	Movement, μm	% of ZAS movement[b]
1:10	6	45	
1:100	6	64	42
1:1000	6	61	
ZAS	6	73	

[a] Standard crude extract.
[b] ZAS = Zymosan-activated serum.

Table 2. Lung cell chemotactic activity following prechallenge with bract extracts.

Prechallenge	Chemotactic activity, no. PMN migrating
Saline (control)	50.46 ± 4.22
Bract	
Responders	75.60 ± 14.84[a]
Nonresponders	74.67 ± 7.23[a]

[a] $p < 0.05$ vs. control.

chemotactic factor was significant (p <0.05) compared with controls. Controls were cells from subjects pre-challenged with saline. The release of chemotactic factor by bract challenged subjects did not correlate with the subject's airway constrictor response. As shown in Table 2, no difference was found between chemotactic activity of cells from responders and nonresponders. Thus, these data cannot confirm that chemotaxis is a specific mechanism of the acute airway constrictor response to cotton bract.

In additional studies, attempts were made to look for release of other cell factors and mediators in the lung's response to cotton bract exposure. Experiments to determine if cells release superoxide anion in response to bract exposure were carried out with the lung cells from bract prechallenged subjects obtained by lavage and subsequently put to culture (22). Cells were stimulated with PMA (phorbol myristate acetate) and the culture medium analyzed for O_2^- 20 min later. Cells from responders released an increased amount of O_2^-. This release by responders (20.76 nmole O_2^-) was significantly higher than that from nonresponders (4.04 nmole) and from controls and showed a significant correlation (p <0.01) with the acute airway constrictor response. The possibility may exist therefore, that oxidant injury may be involved in the constrictor response.

The search for cell mediators in the fluid portion of the bronchoalveolar lavage fluid is only at the preliminary stage and to date has yet to be rewarding. First attempts were made to assay for the presence of leukotrienes B and C. Lavage fluid was obtained from bract-prechallenged volunteers, and cells were removed by centrifugation prior to leukotriene assay. The lavage fluid was concentrated by freeze-drying prior to analysis. Results from radioimmune assay for LTB and LTC are given in Table 3. Only small amounts of leukotriene were detected in the bract prechallenged fluids. The assay for LTC showed essentially no difference between bract-prechallenged fluids and controls. The LTB assay showed a small but somewhat higher content of leukotriene B-like material in the bract-prechallenged fluids versus control fluids, although the number of samples thus far assayed is too small for statistical verification. The bract extract itself, as will be discussed below, was found to be positive in the leukotriene B radioimmune assay and may account for the increase in LTB seen with the bract-prechallenged samples. Regardless, the amounts detected appear to be too small to implicate release of leukotriene from lung cells as a mechanism in the acute airway constrictor response to cotton bracts. Bronchoalveolar lavage in our studies was performed approximately 1 hr after bract inhalation, and our results, therefore, may not detect leukotriene released at earlier or later times during the response.

In vitro experiments with animal lung (23,24) and also with autopsy specimens of human lung (25) have shown that these tissues release histamine when cotton dust or bract extracts are added to the culture medium. As suggested by these studies, histamine released from mast cells may be a mediator in the response mechanism. The bract extract itself contains negligible amounts of histamine. Although several investigators (26) have studied the effect of cotton on histamine release, none have measured histamine release *in vivo* in the primary target organ itself, the lung. We have not yet completed histamine measurements on the lung lavage fluid obtained from volunteers prechallenged with bract extracts. We have looked for an increased histamine content in the urine of volunteers 3 hr after they inhaled cotton bract extract. A sensitive HPLC technique was used to assay the urine for histamine. The results (shown in Table 4) were negative: we found no increase in urinary histamine following inhalation of the bract extract. A better choice for urinary measurements might have been the metabolite of histamine, MIAA (methyl imidazoleacetic acid) content rather than histamine itself, but until more studies are completed, we do not have direct evidence for histamine release *in vivo*.

Contraction of Guinea Pig Smooth Muscle *In Vitro*

Several laboratories have reported that cotton dust or bract extracts contract smooth muscle preparations *in vitro* (27–29). We have confirmed this action using partially purified bract extracts. Strips of guinea pig lung parenchyma and guinea pig longitudinal muscle isolated from the ileum by the Rang procedure and placed in an organ bath contracted on addition of bract extract to the

Table 3. Leukotriene assay of human lung lavage fluid.

	LTC, total ng/sample	LTB, total ng/sample
Bract-prechallenged samples		
1	7.8	6.8
2	4.4	5.4
3	3.8	6.0
Control samples[a]		
1	4.2	3.2
2	3.8	3.0

[a] Saline prechallenged.

Table 4. Bract inhalation challenge and urinary histamine.

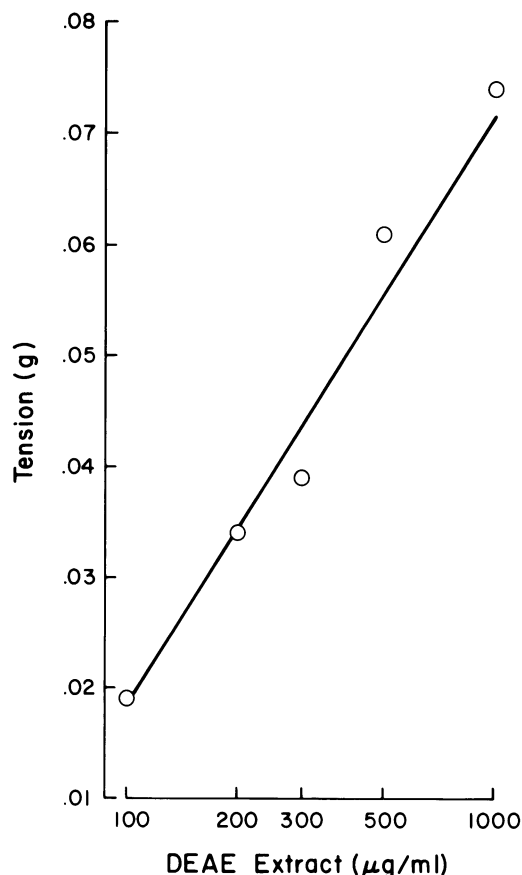|		Urinary histamine, ng/mL	
	MEF 40%	Before challange	After challenge
Responders			
B	44	15.9	8.7
D	35	<2.0	2.9
P	32	87.1	92.9
J	33	5.7	4.4
S	33	40.0	2.9
H	42	174.3	130.0
Nonresponders			
J	13	5.8	5.8
B	14	94.0	41.0
R	13	140.0	254.0
P	0	<2.0	<2.0
H	18	451.0	200.0
S	7	140.0	90.0

FIGURE 3.   Contractile response to guinea pig ileal longitudinal muscle to cotton bract. Force of contraction (g tension) is plotted against concentration of bract extract. Bract extract was a purified preparation, the DEAE preparation. Longitudinal muscle was mounted in an organ bath. Concentration of extract is that in the organ bath.

bathing medium. Both contracted in a dose–response fashion, and Figure 3 is the dose–response curve for the ileal longitudinal muscle. Atropine, an acetylcholine blocker, had no effect on the response, but mepyramine, an H–1 blocker, blocked the contraction, suggesting that a histamine-type response may be involved.

Our ileal longitudinal muscle strip preparation was examined histologically to learn what kinds of cells are present that might release histamine and/or other mediators. Basophils were not seen. Mast cells were seen—an average of two per visualized field. These numbers are about the same as seen in the skin of the guinea pig. The contraction in response to bract extract was not diminished by repeated stimulation/wash-out cycles as one might have expected if degranulation of mast cells is involved in the contraction mechanism. However, before an indirect action involving mast cells can be excluded, more rigorous experiments are necessary.

Airway-Constricting Substances in Cotton Bract Extracts

The possibility remains that the response to cotton bract may involve both direct and indirect modes of ac-

tion. If a direct action exists, are there known constricting agents present in cotton bract extracts. Partial chemical characterization of crude and partially purified bract extracts has been described previously (7,30). These properties of the unknown airway constrictor in bracts include solubility in water, a small molecular weight, a polar and nonionic nature, properties not inconsistent with a small peptide or numerous other compounds. The concentration of histamine in crude bract extracts is only 9 μm/mL—an amount far too low to cause airway constriction when inhaled even in the most sensitive individuals.

Endotoxin which originates from gram-negative bacteria normally found on cotton plants is another known airway constricting agent. It has been shown to be present in the airborne dust of cotton mills and in crude bract extract. Its concentration in mill dust has been reported to correlate better with worker's byssinotic symptoms than the total dust concentration (31,32). However, we found no correlation between the endotoxin content of crude bract extracts and the airway constrictor response following inhalation of these extracts by volunteers. Furthermore, purified bract extracts which had had essentially all endotoxin removed (less than 1 ng/mL remaining) retained their airway-constricting activity (6). Thus cotton bracts contain an agent other than endotoxin which causes airway constriction in humans. When both this
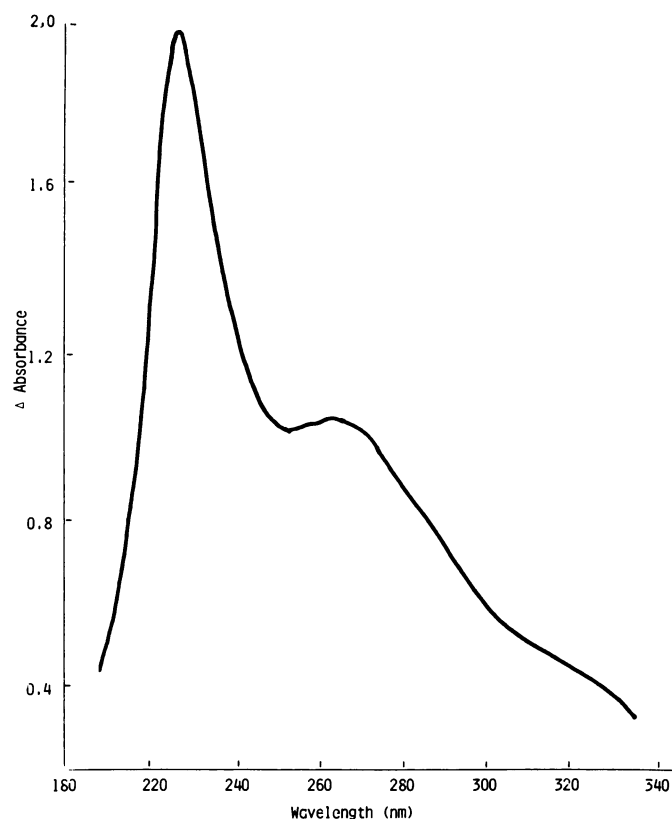

FIGURE 4.   Ultraviolet absorbance spectrum of purified cotton bract extract.

Table 5. Leukotriene assay of cotton bract extracts.

	LTC, ng/mL	LTB, ng/mL
SCE, (60 mg/mL)[a]	0.8	1.9
Purified extract (13.5 mg/mL)	0	1.7

[a] Standard crude extract.

unknown constricting agent and endotoxin, even at sub-levels, are present together, as undoubtedly they are in mill dust, they may act synergistically.

Another possibility is a leukotriene-like compound. Leukotrienes have not yet been identified from plant sources; however, arachidonic acid pathways including lipoxygenase are known (33). Our purified cotton bract extracts exhibit spectral and fluorescent properties similar to leukotrienes isolated from mammalian sources. Figure 4 shows the UV spectrum of the bract extract. It exhibits a triplet peak centered at 262 nm, somewhat to the blue of the known leukotrienes which have their triplet maximum at 270 nm for LTB and at 280 nm for LTC and D. Bract extract fluoreses at 430 nm, as do the known leukotrienes, but its excitation maximum is at 320 nm, not 270 nm as for the known leukotrienes.

Cotton bract extracts tested by radioimmune assay gave a positive reaction for LTB. The results are shown in Table 5. Both the crude and partially purified bract extracts gave the same assay value of about 2 ng/mL LTB-like material. Although the crude extract also gave a positive LTC reaction, the purified extract showed no LTC-like reaction. Either bract extracts interfere with the radioimmune assay or bract contains a leukotriene B-like component. The presence of a LTB-like component in bract extracts could explain their airway constrictor effect and their chemotactic activity. Leukotriene B has been shown to be a chemoattractant. Our data for a bract LTB-like component is suggestive only at this stage. The polar nature and the insolubility of bract extract components in organic solvents do not support the suggestion of a lipid-like compound such as a leukotriene. More rigorous data is needed to confirm the presence of a leukotriene B-like component in cotton bract.

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